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(54) Title: TREATMENT OF DOUGH WITH A LIPOXYGENASE AND A LIPOLYTIC ENZYME

(57) Abstract: The addition of a lipoxygenase and a lipolytic enzyme active on polar lipids to a dough has a synergistic effect on the volume and/or crumb color of an edible product made by leavening and heating the dough, e.g. by baking or steaming.

13

TREATMENT OF DOUGH WITH A LIPOXYGENASE AND A LIPOLYTIC ENZYME

FIELD OF THE INVENTION

The present invention relates to a process for preparing an edible product by leavening and heating the dough, e.g. by baking or steaming. More particularly, it relates to such a process for preparing a product with an increased volume and/or improved crumb color (whiteness).

BACKGROUND OF THE INVENTION

In the preparation of edible products by leavening and heating a dough, it is generally desirable to increase the volume of the product and to improve the crumb color (make the crumb whiter).

WO 9826057 and US 4567046 disclose the addition of a phospholipase to dough. JP 55153549A discloses addition of a lipase and a lipoxygenase to flour. WO 9953769 and WO 2002094123 disclose the addition of enzymes to dough.

SUMMARY OF THE INVENTION

The inventors have found that the addition of a lipoxygenase and a lipolytic enzyme active on polar lipids to a dough has a synergistic effect on the volume and/or crumb color of an edible product made by leavening and heating the dough, e.g. by baking or steaming.

Accordingly, the invention provides a process for preparing an edible product, comprising adding a lipoxygenase and a lipolytic enzyme active on polar lipids to a dough, leavening, and heating the dough, wherein the lipoxygenase and the lipolytic enzyme are added in amounts producing a synergistic effect on the volume of the edible product.

The invention also provides a composition for use in the process.

DETAILED DESCRIPTION OF THE INVENTION

Lipoxygenase

The lipoxygenase (EC 1.13.11.12) is an enzyme that catalyzes the oxygenation of poly-unsaturated fatty acids such as linoleic acid, linolenic acid and arachidonic acid, which contain a *cis,cis*-1,4-pentadiene unit and produces hydroperoxides of these fatty acids. The lipoxygenase of the invention is able to oxidize substrates containing a *cis-cis*-pentadienyl moiety. Thus, it may act on polyunsaturated fatty acids such as linoleic acid (18 carbon atoms, 2 double bonds), linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (EPA, 20:5) and/or docosahexaenoic acid (DHA, 22:6).

The lipoxygenase may be a 9-lipoxygenase with the ability to oxidize the double bond

between carbon atoms 9 and 10 in linoleic acid and linolenic acid, or it may be a 13-lipoxygenase with the ability to oxidize the double bond between carbon atoms 12 and 13 in linoleic acid and linolenic acid.

The lipoxygenase may be from animal, plant or microbial source. A plant lipoxygenase may be from plants of the pulse family (*Fabaceae*), soybean (lipoxygenases 1, 2 and 3), cucumber, or barley. A microbial lipoxygenase may be from a yeast such as *Saccharomyces cerevisiae*, a thermophilic actinomycete such as *Thermoactinomyces vulgaris* or *Thermomyces*, e.g. *T. lanuginosus*, or from fungi.

A fungal lipoxygenase may be derived from *Ascomycota*, particularly *Ascomycota incertae sedis* e.g. *Magnaporthaceae*, such as *Gaeumannomyces* or *Magnaporthe*, or anamorphic *Magnaporthaceae* such as *Pyricularia*, or alternatively anamorphic *Ascomycota* such as *Geotrichum*, e.g. *G. candidum*. The fungal lipoxygenase may be from *Gaeummanomyces graminis*, e.g. *G. graminis var. graminis*, *G. graminis var. avenae* or *G. graminis var. tritici*, (WO 0220730) or *Magnaporthe salvinii* (PCT/DK 02/00251). Also, a fungal lipoxygenase may be from *Fusarium* such as *F. oxysporum* or *F. proliferatum*, or *Penicillium sp.*

The lipoxygenase may be used at a dosage of 0.01-10 mg of enzyme protein per kg of flour, particularly 0.1-5 mg/kg, e.g. 0.2-1 mg/kg.

Lipolytic enzyme active on polar lipids

The invention uses a lipolytic enzyme which is capable of hydrolyzing carboxylic ester bonds in polar lipids such as phospholipids and/or galactolipids, i.e. having phospholipase and/or galactolipase activity. Thus, the lipolytic enzyme may have phospholipase A1 or A2 activity (EC 3.1.1.32 or 3.1.1.4), i.e. hydrolytic activity towards one or both carboxylic ester bonds in phospholipids such as lecithin. Further, the lipolytic enzyme may have galactolipase activity (EC 3.1.1.26), i.e. hydrolytic activity on carboxylic ester bonds in galactolipids such as DGDG (digalactosyl diglyceride).

The lipolytic enzyme may or may not have lipase activity (activity on triglycerides, EC 3.1.1.3). It may have a higher activity on polar lipids than on triglycerides.

The lipolytic enzyme may be of animal origin, e.g. from pancreas, snake venom or bee venom, or it may be of microbial origin, e.g. from filamentous fungi, yeast or bacteria, such as *Aspergillus or Fusarium, e.g. A. niger, A. oryzae* or *F. oxysporum*, e.g. the enzymes described in WO 9826057, WO 0200852. Also, the variants described in WO 0032758 may be used, e.g. a variant of *Thermomyces lanuginosus* lipase having phospholipase and/or galactolipase activity.

The lipolytic enzyme may be used at a dosage of 0.01-10 mg of enzyme protein per kg of flour, particularly 0.1-5 mg/kg, e.g. 0.2-1 mg/kg.

Synergistic effect

The combination of the lipoxygenase and the lipolytic enzyme has a synergistic effect on volume and/or crumb color of an edible product made by leavening and heating the dough.

Synergy may be determined by making doughs or baked products with addition of the two enzymes separately and in combination, and comparing the effects; synergy is indicated when the combination produces a better effect than each enzyme used separately.

The comparison may be made between the combination and each enzyme alone at double dosage (on the basis of enzyme protein or enzyme activity). Thus, synergy may be said to occur if the effect of 0.5 mg of enzyme A + 1.0 mg of enzyme B is greater than the effect with 1.0 mg of enzyme A and also greater than the effect with 2.0 mg of enzyme B.

Alternatively, the comparison may be made with equal total enzyme dosages (as pure enzyme protein). If the effect with the combination is greater than with either enzyme alone, this may be taken as an indication of synergy. As an example, synergy may be said to occur if the effect of 0.5 mg of enzyme A + 1.0 mg of enzyme B is greater than with 1.5 mg of enzyme A or B alone.

Dough

The dough is leavened e.g. by adding chemical leavening agents or yeast, usually *Saccharomyces cerevisiae* (baker's yeast).

The dough generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, rye meal, rye flour, oat flour, oat meal, sorghum meal, sorghum flour, rice flour, potato meal, potato flour or potato starch.

The dough may be fresh, frozen or par-baked.

The dough may be a laminated dough.

The dough may also comprise other conventional dough ingredients, e.g.: proteins, such as milk powder and gluten; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough may comprise fat (triglyceride) such as granulated fat or shortening.

The dough may further comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin.

Edible product

The process of the invention is used for preparing a an edible product by leavening and heating a dough, e.g. by baking or steaming. The product may be of a soft or a crisp character, either of a white, light or dark type. Examples are steamed or baked bread bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

Enzyme composition

The invention provides a composition (e.g. a baking composition) comprising a lipoxygenase, a phospholipase and optionally an additional enzyme as described below.

The composition may be an enzyme preparation, e.g. in the form of a granulate or agglomerated powder. It may have a narrow particle size distribution with more than 95 % (by weight) of the particles in the range from 25 to 500 μm. Granulates and agglomerated powders may be prepared by conventional methods, e.g. by spraying the amylase onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, e.g. a salt (such as NaCl or sodium sulfate), a sugar (such as sucrose or lactose), a sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy.

The composition may, in addition to enzymes, comprise other baking ingredients, particularly flour. Thus, the composition may be a dough or a flour pre-mix.

Additional enzyme

Optionally, an additional enzyme may be used together with the lipoxygenase and the lipolytic enzyme.

The additional enzyme may be an amylase, a cyclodextrin glucanotransferase, a protease or peptidase, in particular an exopeptidase, a transglutaminase, a lipase, a phospholipase, a cellulase, a hemicellulase, a glycosyltransferase, a branching enzyme (1,4-α-glucan branching enzyme) or a second oxidoreductase. The additional enzyme may be of any origin, including mammalian and plant, and preferably of microbial (bacterial, yeast or fungal) origin.

The amylase may be from a fungus, bacterium or plant. It may be a maltogenic alpha-amylase (EC 3.2.1.133), e.g. from *B. stearothermophilus*, an alpha-amylase, e.g. from *Bacillus*, particularly *B. licheniformis* or *B. amyloliquefaciens*, a beta-amylase, e.g. from plant (e.g. soy bean) or from microbial sources (e.g. *Bacillus*), a glucoamylase, e.g. from *A. niger*, or a fungal alpha-amylase, e.g. from *A. oryzae*.

The hemicellulase may be a pentosanase, e.g. a xylanase which may be of microbial origin, e.g. derived from a bacterium or fungus, such as a strain of *Aspergillus*, in particular of *A.*

aculeatus, *A. niger*, *A. awamori*, or *A. tubigensis*, from a strain of *Trichoderma*, e.g. *T. reesei*, or from a strain of *Humicola*, e.g. *H. insolens*.

The protease may be from *Bacillus*, e.g. *B. amyloliquefaciens*.

The second oxidoreductase may be a glucose oxidase, a hexose oxidase, a peroxidase, or a laccase.

EXAMPLES

Example 1

1 kg flour doughs were prepared by a straight dough procedure with addition of phospholipase from *F. oxysporum* and lipoxygenase (LOX) from *M. salvinii* as shown in the table below. The LU activity unit is defined in WO 0032758.

The doughs were leavened and baked, and the specific volume and crumb properties were evaluated for bread baked from each dough. Crumb properties were evaluated by a panel using a scale from 0 to 10 taking the control as 5, as follows:

Uniform: 0=uneven, 10=very uniform

Grain: 0= open, 10=fine

Cell wall: 0= thick, 10=thin

Cell form: 0=round, 10=elongate

Crumb color: 0=dark, 10 =white

	Invention	Control	Reference		
Phospholipase, LU/kg	500		500		
LOX, mg/kg	0.2			0.2	
Soy flour, % by weight					0.5
Sp. Vol. (ml/g)	5.06	4.31	4.78	4.45	4.36
Sp. Vol. (%)	117	100	111	103	101
Crumb evaluation (Ext. proof)					
Uniform	7	5	7	3	4
Grain	7	5	7	2	4
Cell Wall	7	5	7	4	4
Cell Form	7	5	7	2	6
Crumb Color	7	5	6	6	8

The results show that soy flour has no impact on volume, but the crumb color (whiteness) is improved by soy flour.

LOX alone has no impact on volume, and the crumb color is slightly improved compared to the control.

The phospholipase alone gives clear volume and crumb structure improvements

LOX in combination with the lipase has a synergistic effect on volume, and crumb color is also improved compared to the phospholipase or LOX alone.

CLAIMS

1. A process for preparing an edible product, comprising adding a lipoxygenase and a lipolytic enzyme active on polar lipids to a dough, leavening, and heating the dough, wherein the lipoxygenase and the lipolytic enzyme are added in amounts producing a synergistic effect on the volume of the edible product.

2. A process of preparing a baked product comprising:
a) adding to a dough a lipoxygenase and a lipolytic enzyme active on polar lipids, and
b) baking the dough,
wherein the lipoxygenase and the lipolytic enzyme are added in amounts producing a synergistic effect on the volume or the crumb color of the baked product.

3. A composition comprising: a lipoxygenase and a lipolytic enzyme active on polar lipids wherein the lipoxygenase and the lipolytic enzyme are added in amounts producing a synergistic effect on the volume or the crumb color of the baked product.

4. The composition of the preceding claim which further comprises flour.

5. The composition of the preceding claim which is a dough, a flour composition, or a flour pre-mix.

6. A method of increasing the volume or the crumb color of a baked product comprising:
a) adding to a dough a lipoxygenase and a lipolytic enzyme which is active on polar lipids and on triglycerides,
b) baking the dough to prepare a baked product, and
c) measuring the volume or the crumb color of the baked product.

INTERNATIONAL SEARCH REPORT

Internati ilication No
PCT/DK 03/00460

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A21D8/04 C12N9/20 C12N9/02 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A21D C12N C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, MEDLINE, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 02 19828 A (NOVOZYMES AS) 14 March 2002 (2002-03-14) claim 8 ---	1-6
P,A	WO 02 086114 A (NOVOZYMES AS ;TAKAGI SHINOBU (JP); SUGIO AKIKO (US)) 31 October 2002 (2002-10-31) claim 11 ---	1-6
Y	T. GALLIARD: "Hydrolytic and Oxidative Degradation of Lipids During Storage of Wholemeal Flour: Effects of Bran and Germ Components " JOURNAL OF CEREAL SCIENCE, vol. 4, 1986, pages 179-192, XP002255190 figures 1-6 --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2003	05. 11. 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	FERNANDO FARIETA / ELY

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INTERNATIONAL SEARCH REPORT

Internati oplication No
PCT/DK 03/00460

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 198106 Derwent Publications Ltd., London, GB; Class D11, AN 1981-08437D XP002255191 & JP 55 153549 A (MEIJI SEIKA KAISHA ET AL), 29 November 1980 (1980-11-29) abstract	1-6
A	WO 02 03805 A (CHRISTENSEN LUISE ;SPENDLER TINA (DK); BUDOLFSEN GITTE (DK); NOVOZ) 17 January 2002 (2002-01-17) claims 1-14	1-6
A	WO 00 32758 A (SHAMKANT ANANT PATKAR ;BORCH KIM (DK); PETRI ANDREAS (DK); VINDJE) 8 June 2000 (2000-06-08) claim 64	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 03/00460

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-5
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-5

Present claims 1-5 relate to processes and compositions defined by reference to a desirable characteristic or property, namely "to be added in amounts producing a synergistic effect".

The claims cover all processes and compositions having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and / or disclosure within the meaning of Article 5 PCT for only a very limited number of such processes and compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the processes and compositions by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the processes and compositions prepared in example 1.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK 03/00460

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0219828	A	14-03-2002	AU	8381701 A	22-03-2002
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